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Regulation of the pp72^{syk} protein tyrosine kinase by platelet integrin $\alpha_{IIb}\beta_3$

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pp72^{syk} is essential for development and function of several hematopoietic cells, and it becomes activated through tandem SH2 interaction with ITAM motifs in immune response receptors. Since Syk is also activated through integrins, which do not contain ITAMs, a CHO cell model system was used to study Syk activation by the platelet integrin, $\alpha_{IIb}\beta_3$. As in platelets, Syk underwent tyrosine phosphorylation and activation during CHO cell adhesion to $\alpha_{IIb}\beta_3$ ligands, including fibrinogen. This involved Syk autophosphorylation and the tyrosine kinase activity of Src, and it exhibited two novel features. Firstly, unlike $\alpha_{IIb}\beta_3$-mediated activation of pp125^{FAK}, Syk activation could be triggered by the binding of soluble fibrinogen and abolished by truncation of the α_{IIb} or β_3 cytoplasmic tail, and it was resistant to inhibition by cytochalasin D. Secondly, it did not require phosphorylated ITAMs since it was unaffected by disruption of an ITAM-interaction motif in the SH2(C) domain of Syk or by simultaneous overexpression of the tandem SH2 domains. These studies demonstrate that Syk is a proximal component in $\alpha_{IIb}\beta_3$ signaling and is regulated as a consequence of intimate functional relationships with the $\alpha_{IIb}\beta_3$ cytoplasmic tails and with Src or a closely related kinase. Furthermore, there are fundamental differences in the activation of Syk by $\alpha_{IIb}\beta_3$ and immune response receptors, suggesting a unique role for integrins in Syk function.

Keywords: CHO cells/integrin/platelet/protein tyrosine kinase/Src/Syk

Introduction

Integrin $\alpha_{IIb}\beta_3$ plays an essential role in hemostasis and thrombosis by mediating platelet adhesion and aggregation. These functions are regulated by platelet agonists and antagonists, which increase or decrease $\alpha_{IIb}\beta_3$ affinity/avidity for adhesive ligands through a process known as 'inside–out' signaling. In turn, ligand occupancy and clustering of $\alpha_{IIb}\beta_3$ trigger 'outside–in' signals that influence cytoskeletal events during platelet aggregation and clot retraction (Clark and Brugge, 1995). Integrin signaling is also involved in mediating anchorage-dependent growth, differentiation and survival of nucleated cells. Accordingly, there have been intense efforts to define the precise relationships between integrins and cellular signaling pathways (Hynes, 1992; Clark and Brugge, 1995; Schwartz *et al.*, 1995; Juliano, 1996; Sastry and Horwitz, 1996; Yamada and Geiger, 1997).

Studies in platelets have highlighted a role for protein tyrosine phosphorylation in integrin-mediated outside–in signaling (Clark and Brugge, 1995). After $\alpha_{IIb}\beta_3$ interacts with soluble or immobilized fibrinogen, there is an immediate increase in tyrosine phosphorylation of several substrates, one of which is the non-receptor protein tyrosine kinase, pp72^{syk} (Haimovich *et al.*, 1993; Huang *et al.*, 1993; Clark *et al.*, 1994). As platelets then begin to aggregate or spread, activation of the tyrosine kinase pp125^{FAK} occurs, coincident with the redistribution of a number of proteins to the Triton X-100-insoluble core cytoskeleton, including $\alpha_{IIb}\beta_3$, Syk, FAK and pp60^{src} (Clark and Brugge, 1993; Fox *et al.*, 1993; Haimovich *et al.*, 1993; Clark *et al.*, 1994). Toward the later stages of aggregation, tyrosine dephosphorylation occurs coincident with activation and/or redistribution of protein tyrosine phosphatases (Frangione *et al.*, 1993; Ezumi *et al.*, 1995). Integrin signaling is clinically significant because all of these platelet responses are deficient in individuals who bleed due to heritable mutations in the cytoplasmic tail of the β_3 integrin subunit (Chen *et al.*, 1994b; Wang *et al.*, 1997). Despite this apparent requirement for the integrin cytoplasmic tail, it is not clear how fibrinogen binding to $\alpha_{IIb}\beta_3$ is coupled to activation of the tyrosine kinases and phosphatases. It would be particularly useful to understand how one of the earliest events, Syk tyrosine phosphorylation, takes place. In this regard, it is worth noting that Syk also becomes activated in response to ligation of β_1 integrins in neutrophils and β_2 integrins in monocytic cells (Lin *et al.*, 1995; Yan *et al.*, 1997).

Syk contains two N-proximal SH2 domains, two interdomain spacer regions, a catalytic domain and a C-terminal tail, and it is restricted to hematopoietic cells (Chan *et al.*, 1992; Müller *et al.*, 1994). It is homologous to ZAP-70, whose expression is limited even further to T lymphocytes and NK cells. Syk becomes activated in particular hematopoietic cells in response to ligation of the B cell receptor, the T cell receptor or several Fc receptors, and it is clearly implicated in lymphocyte development and activation and mast cell degranulation (Minoguchi *et al.*, 1994; Cambier, 1995; Cheng *et al.*, 1995; Turner *et al.*, 1995; Qian and Weiss, 1997). In these cases, receptor engagement stimulates tyrosine phosphorylation of 'immune receptor tyrosine activation' motifs (ITAMs) in the receptor by a Src family kinase. Syk activation occurs when the tandem

SH2 domains engage a dually-phosphorylated ITAM, and activation appears to occur through a chain reaction mechanism involving tyrosine phosphorylation in the activation loop of the catalytic domain (Kurosaki *et al.*, 1995; Rowley *et al.*, 1995; Shiue *et al.*, 1995a; Kimura *et al.*, 1996; El-Hillal *et al.*, 1997). It is difficult to envisage how this mechanism would apply to integrins since, except for the β_4 subunit, their cytoplasmic tails do not contain ITAMs. On the other hand, platelets do contain at least two ITAM-containing proteins, the FcγRIIA receptor (Chacko *et al.*, 1996) and the common FcRγ subunit (Asselin *et al.*, 1997). Indeed the latter has been implicated in platelet responses to collagen, although current evidence suggests this does not occur directly through an integrin (Keely and Parise, 1996; Asselin *et al.*, 1997; Ichinohe *et al.*, 1997; Poole *et al.*, 1997). Nonetheless, it remains formally possible that $\alpha_{IIb}\beta_3$ relies on an ITAM-containing protein for activation of Syk.

The purpose of the present studies was to characterize the mechanism of Syk activation by $\alpha_{IIb}\beta_3$. Since platelets are not amenable to genetic manipulation and possess integrin-dependent and integrin-independent pathways of Syk activation (Clark *et al.*, 1994; Keely and Parise, 1996; Asselin *et al.*, 1997; Yanabu *et al.*, 1997), we sought to establish a more tractable model system. Chinese hamster ovary (CHO) cells were selected because they contain Src family kinases (Cary *et al.*, 1996) but are unlikely to contain endogenous Syk, and stable transfectants expressing human $\alpha_{IIb}\beta_3$ undergo a typical outside–in signaling response—tyrosine phosphorylation of FAK—upon adhesion to $\alpha_{IIb}\beta_3$ ligands (Leong *et al.*, 1995). By means of transient transfection, we have now been able to reconstitute $\alpha_{IIb}\beta_3$-dependent activation of Syk in these cells and to define the roles of integrin cytoplasmic tails, ITAMs and other protein tyrosine kinases, such as Src and FAK, in this process.

Results

Integrin activation of Syk in a CHO cell model system

In platelets, Syk becomes activated within seconds of fibrinogen binding to integrin $\alpha_{IIb}\beta_3$. To study how Syk is regulated by $\alpha_{IIb}\beta_3$, human Syk was transiently-transfected into a CHO cell line (A5) that stably expresses human $\alpha_{IIb}\beta_3$, and the cells were studied 48 h later. Preliminary studies indicated that there was no immunologically detectable Syk in mock-transfected CHO cells, whereas cells transfected with the EMCV/Syk expression plasmid expressed Syk in a dose-dependent fashion.

To determine whether Syk could undergo tyrosine phosphorylation in response to adhesion of A5 cells to an $\alpha_{IIb}\beta_3$ ligand, Syk transfectants were incubated for 60 min over a BSA matrix to which the cells did not bind or a fibrinogen matrix to which ⩾80% of the cells bound. At lower input levels of Syk (plasmid DNA ⩽1 μg), A5 cells maintained in suspension over the BSA matrix exhibited little or no tyrosine phosphorylation of Syk. On the other hand, fibrinogen-adherent cells exhibited a several-fold increase in Syk phosphorylation (Figures 1A and B). At plasmid DNA levels >1 μg, some tyrosine phosphorylation of Syk was evident even in the suspended cells, although to a lesser degree than in adherent cells (Figure

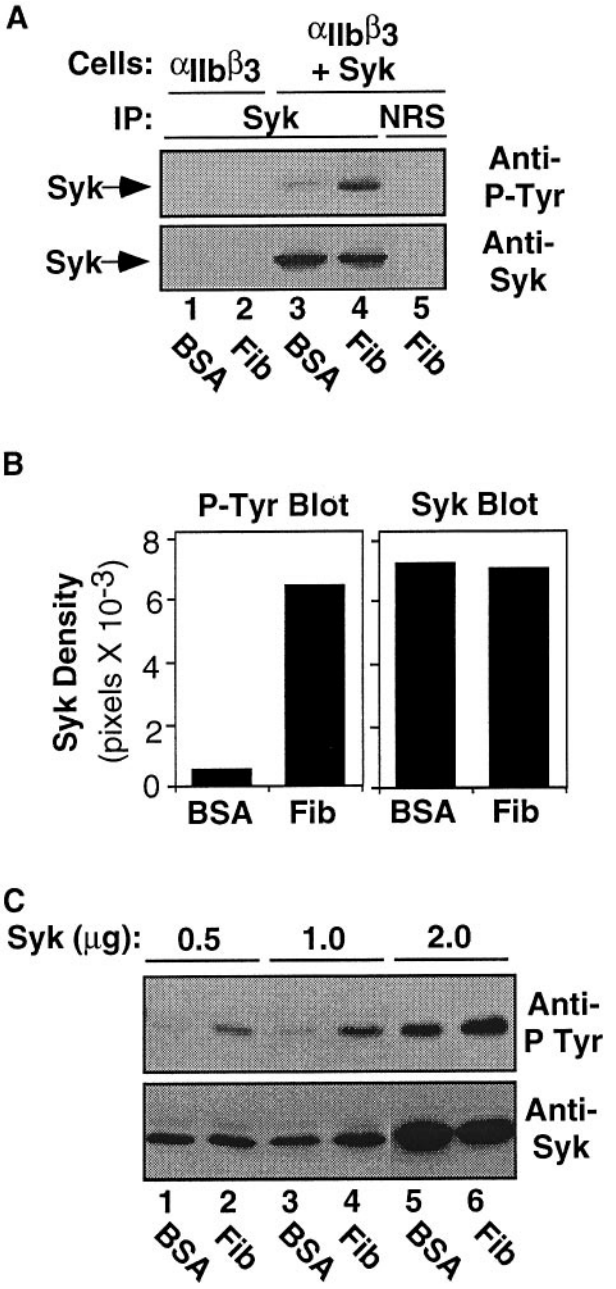

Fig. 1. Effect of CHO cell adhesion via $\alpha_{IIb}\beta_3$ on tyrosine phosphorylation of Syk. As described in Materials and methods, A5 CHO cells expressing $\alpha_{IIb}\beta_3$ were transiently-transfected with Syk or vector control DNA. Forty-eight hours later, the cells were washed and maintained in suspension for 60 min at 37°C (BSA) or allowed to attach to a culture dish coated with fibrinogen (Fib). Cells were lysed in RIPA buffer and the extent of Syk tyrosine phosphorylation was analyzed on Western blots of Syk immunoprecipitates. Blots were reprobed with an anti-Syk antibody to assess the amount of Syk loaded onto each gel lane. In (**A**) 1.0 μg of Syk or control plasmid was transfected per dish. Lane 5 represents a control immunoprecipitation using normal rabbit serum (NRS) The data in this and all other figures represent the results of a single experiment that were confirmed in three or more additional, independent experiments. In (**B**) the band densities from the Syk transfectants in A were quantitated by densitometry. In (**C**) the effect of different amounts of Syk expression on Syk tyrosine phosphorylation was examined. The values given for Syk represent the amount of plasmid DNA transfected per dish.

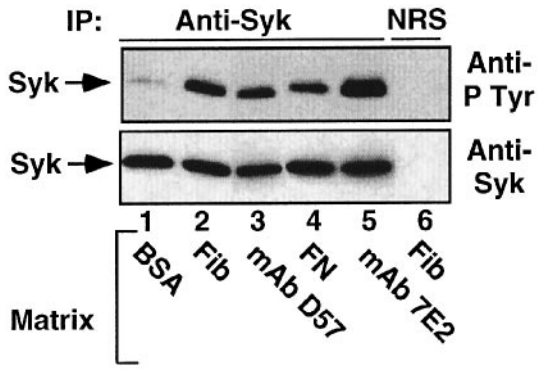

Fig. 2. Effect of CHO cell adhesion to ligands for $\alpha_{IIb}\beta_3$ or β_1 integrins on tyrosine phosphorylation of Syk. A5 CHO cell transfectants were maintained in suspension for 60 min (BSA, lane 1) or allowed to attach to $\alpha_{IIb}\beta_3$ ligands (fibrinogen, lane 2; anti-$\alpha_{IIb}\beta_3$ monoclonal antibody D57, lane 3) or β_1 ligands (fibronectin, FN, lane 4; anti-β_1 monoclonal antibody 7E2, lane 5). Then tyrosine phosphorylation of Syk was assessed.

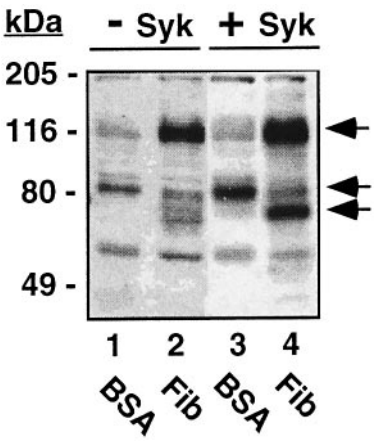

Fig. 3. Tyrosine phosphorylation of proteins in CHO cells transfected with Syk. A5 cells were transfected with Syk (+ Syk) or a control plasmid (– Syk) and 48 h later incubated in suspension (BSA) or allowed to attach to fibrinogen (Fib) for 60 min. Total cell lysates (20 μg/lane) were then examined for tyrosine phosphorylation. The top arrow denotes a broad band at 120–130 kDa that increased in intensity with cell adhesion (lanes 1 versus 2 and 3 versus 4) The middle arrow denotes an unidentified ~80 kDa protein that was reproducibly dephosphorylated during cell adhesion (lanes 1 versus 2 and 3 versus 4) The bottom arrow denotes Syk (lane 4)

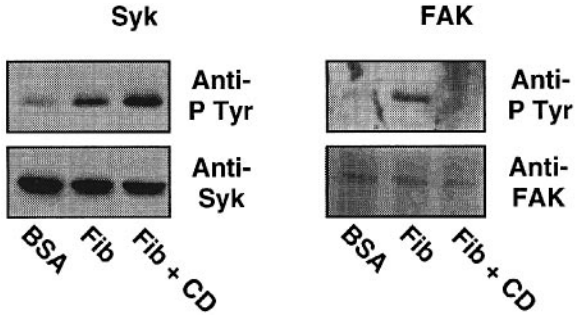

Fig. 4. Effect of cytochalasin D on adhesion-dependent tyrosine phosphorylation of Syk and FAK in CHO cells. Syk-transfected A5 cells were incubated with 10 μM cytochalasin D or DMSO vehicle for 45 min at 37°C. The cells were maintained in suspension (BSA) or allowed to attach to fibrinogen for 60 min and tyrosine phosphorylation of Syk and FAK was determined.

1C). Syk phosphorylation was observed within 15 min of plating cells on fibrinogen, the earliest time point studied. In subsequent studies, therefore, 0.5 μg of Syk plasmid DNA was used. In contrast to these results, no tyrosine phosphorylation of transfected ZAP-70 was observed in suspended or adherent CHO cells, despite levels of expression similar to that of Syk (not shown). Thus, adhesion of A5 cells to an $\alpha_{IIb}\beta_3$ ligand induces tyrosine phosphorylation of Syk.

CHO cells express several integrins, including $\alpha_5\beta_1$, a receptor for fibronectin (Bauer *et al.*, 1992). To determine whether Syk was coupled to integrins other than $\alpha_{IIb}\beta_3$, the response of Syk transfectants to adhesion to β_1 integrin ligands was studied, both in A5 cells and native CHO cells. The results with A5 cells are shown in Figure 2 but are representative of results with native CHO cells. Cell adhesion to either fibronectin or an anti-β_1 antibody stimulated tyrosine phosphorylation of Syk (lanes 4 and 5). This indicates that Syk can be situated downstream of integrins other than $\alpha_{IIb}\beta_3$, consistent with previous observations in neutrophils and monocytic cells (Lin *et al.*, 1995; Yan *et al.*, 1997).

Additional studies were carried out to determine if the tyrosine phosphorylation of Syk in fibrinogen-adherent A5 cells was triggered soley through $\alpha_{IIb}\beta_3$. First, Syk-transfected native CHO cells that lack $\alpha_{IIb}\beta_3$ did not attach to fibrinogen. Second, A5 cell adhesion to fibrinogen was inhibited $\geq$95% by an $\alpha_{IIb}\beta_3$-selective function-blocking antibody (A2A9, 10 μg/ml), a cyclic peptide (Integrilin, 10 μM), or a peptidomimetic (Ro 43–5054, 10 μM) (not shown). Third, A5 cells were routinely preincubated for 45 min with cycloheximide before the adhesion studies to minimize synthesis of potential integrin ligands. Fourth, results identical to those obtained with fibrinogen-adherent A5 cells in Figures 1 and 2 were obtained with A5 cells adherent to mAb D57, a monoclonal antibody specific for $\alpha_{IIb}\beta_3$ (Figure 2, lane 3). Accordingly, fibrinogen and D57 were used interchangeably as ligands in subsequent experiments to study the mechanism of Syk regulation by $\alpha_{IIb}\beta_3$.

Differential regulation of Syk and FAK by $\alpha_{IIb}\beta_3$

A characteristic feature of outside–in signaling in platelets and A5 CHO cells is tyrosine phosphorylation of pp125^{FAK} (Haimovich *et al.*, 1993; Leong *et al.*, 1995). In fact, a broad tyrosine-phosphorylated band was observed at 120–130 kDa in lysates from fibrinogen-adherent A5 cells but not from cells maintained in suspension (Figure 3), and FAK represented a component of this band (see Figure 4). Therefore Syk and FAK, both prominently involved in integrin signaling in platelets, also function downstream of $\alpha_{IIb}\beta_3$ in CHO cells. Several additional unidentified bands became weakly tyrosine-phosphorylated in fibrinogen-adherent cells, and one at ~80 kDa became dephosphorylated. Aside from the prominent 72 kDa band representing Syk, the overall pattern and extent of tyrosine phosphorylation in adherent A5 cells was not consistently affected by expression of Syk.

Syk and FAK differ in two notable respects in platelets. First, activation of FAK during thrombin-induced platelet aggregation is abolished by 10 μM cytochalasin D, an inhibitor of actin polymerization, whereas activation of Syk is not (Clark *et al.*, 1994). This difference was maintained in the CHO cell system (Figure 4), suggesting that FAK activation requires a degree of actin polymeriz-

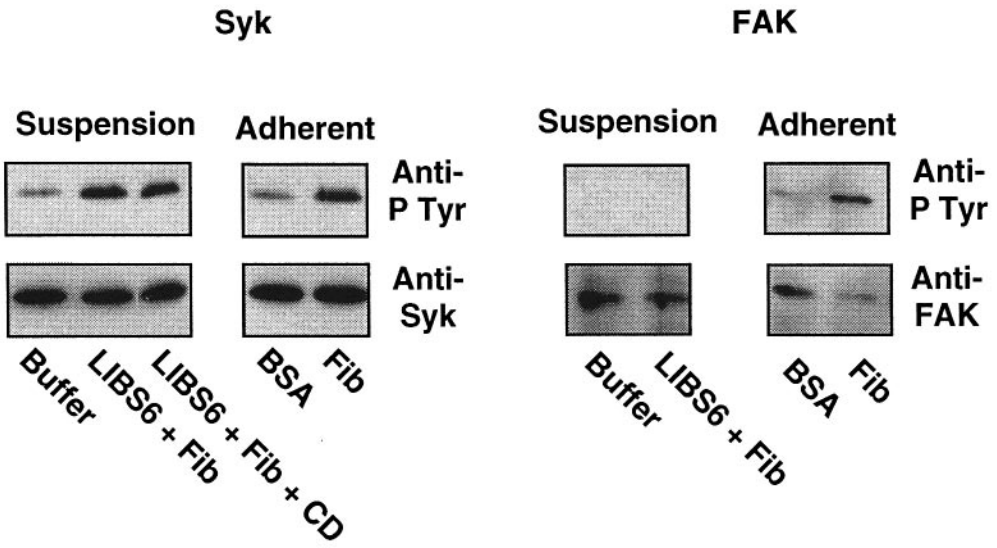

Fig. 5. Binding of soluble fibrinogen to $\alpha_{IIb}\beta_3$ stimulates tyrosine phosphorylation of Syk but not FAK. Syk-transfected A5 cells were incubated in suspension for 30 min in the presence of either (i) DMSO vehicle (buffer), (ii) an activating anti-β_3 Fab fragment (LIBS6; 150 μg/ml) and fibrinogen (Fib; 250 μg/ml) or (iii) LIBS6, fibrinogen and 10 μM cytochalasin D (CD). Then tyrosine phosphorylation of Syk and FAK was assessed. Not shown are controls in which LIBS6 or fibrinogen alone failed to induce Syk phosphorylation, indicating that this response required LIBS6-induced fibrinogen binding to $\alpha_{IIb}\beta_3$. Parallel studies were conducted on adherent cells (Fib) to confirm that FAK was capable of becoming phosphorylated in these cells.

ation not required for Syk. Second, mere binding of soluble fibrinogen to platelets is sufficient to trigger tyrosine phosphorylation of Syk but not FAK (Clark *et al.*, 1994; Shattil *et al.*, 1994). Again, this difference was maintained in the CHO cell system. Tyrosine phosphorylation of Syk but not FAK was observed when fibrinogen binding was induced directly by an 'activating' anti-β_3 antibody, and this was not inhibited by cytochalasin D (Figure 5). These results indicate that Syk phosphorylation is coupled to integrin ligation rather than to some later post-ligand binding event, such as actin polymerization and FAK phosphorylation.

Role of $\alpha_{IIb}\beta_3$ cytoplasmic tails in the regulation of Syk

To evaluate a role for the cytoplasmic tails of α_{IIb} and β_3 in Syk function, studies were performed using established CHO cell lines containing specific cytoplasmic tail deletions or mutations. In $\alpha_{IIb}\Delta 996\beta_3$ cells, 13 of the 20 residues of the α_{IIb} tail have been deleted, while in $\alpha_{IIb}\beta_3\Delta 724$ cells, 39 of the 47 residues of the β_3 tail have been deleted. These deletions were studied to determine whether membrane-distal residues in either cytoplasmic tail were required for Syk phosphorylation. In $\alpha_{IIb}\beta_3$-(Y747F,Y759F) cells, both tyrosines in the β_3 tail have been replaced with phenylalanine. This variant was studied because β_3 becomes tyrosine-phosphorylated during platelet aggregation, producing potential docking sites for SH2 domains (Law *et al.*, 1996b). The level of surface expression of $\alpha_{IIb}\beta_3$ in each variant cell line was comparable to that in A5 cells (Figure 6A), and each adhered normally to $\alpha_{IIb}\beta_3$ ligands. However, the $\alpha_{IIb}\beta_3\Delta 724$ cells exhibit a defect in spreading (Leong *et al.*, 1995).

Similarly to A5 cells, no tyrosine phosphorylation of Syk was observed in any of the mutant cell lines when they were maintained in suspension for 60 min. However, unlike A5 cells which exhibited robust Syk tyrosine phosphorylation during adhesion to mAb D57, little or no Syk phosphorylation was observed in adherent $\alpha_{IIb}\Delta 996\beta_3$ cells or $\alpha_{IIb}\beta_3\Delta 724$ cells (Figure 6B, left-hand panel). These differences could not be explained by variations in Syk expression (Figure 6B). Therefore, membrane-distal residues within both the α_{IIb} and β_3 cytoplasmic tails are required for integrin-dependent tyrosine phosphorylation of Syk. This contrasts with previous studies of FAK phosphorylation which indicated that the β_3 tail is essential but the α_{IIb} tail is dispensible (Leong *et al.*, 1995). Tyrosine phosphorylation of Syk occurred normally in adherent CHO cells expressing $\alpha_{IIb}\beta_3$(Y747F,Y759F), effectively excluding a role for β_3 tail tyrosine residues in the Syk activation process.

Role of ITAMs in Syk regulation by $\alpha_{IIb}\beta_3$

A productive, high-affinity interaction between ITAMs and Syk requires ITAM engagement of both Syk SH2 domains (Chen *et al.*, 1996). As mentioned previously, the cytoplasmic tails of α_{IIb} and β_3 do not contain ITAMs, but platelets contain at least two ITAM-containing proteins, the FcγRIIA receptor (Chacko *et al.*, 1994) and the common FcRγ subunit (Gibbins *et al.*, 1996). To investigate whether a Syk/ITAM interaction was necessary for regulation of Syk by $\alpha_{IIb}\beta_3$, A5 cells were cotransfected with Syk and a truncated form of Syk, Syk(1-330), which contains both SH2 domains and interdomain regions. Syk(1-330) was shown previously to inhibit ITAM-dependent Syk activation in RBL cells (Taylor *et al.*, 1995) and in COS cells stimulated by overexpression of an FcRγ subunit chimera, CD8/γ (K.Zoller and J.S.Brugge, unpublished observations). In contrast to those results, co-expression of Syk(1-330) in A5 cells had no effect on $\alpha_{IIb}\beta_3$-dependent tyrosine phosphorylation of Syk (Figure 7A). This failure was not due to insufficient expression of Syk(1-330) because it was readily detected in Western

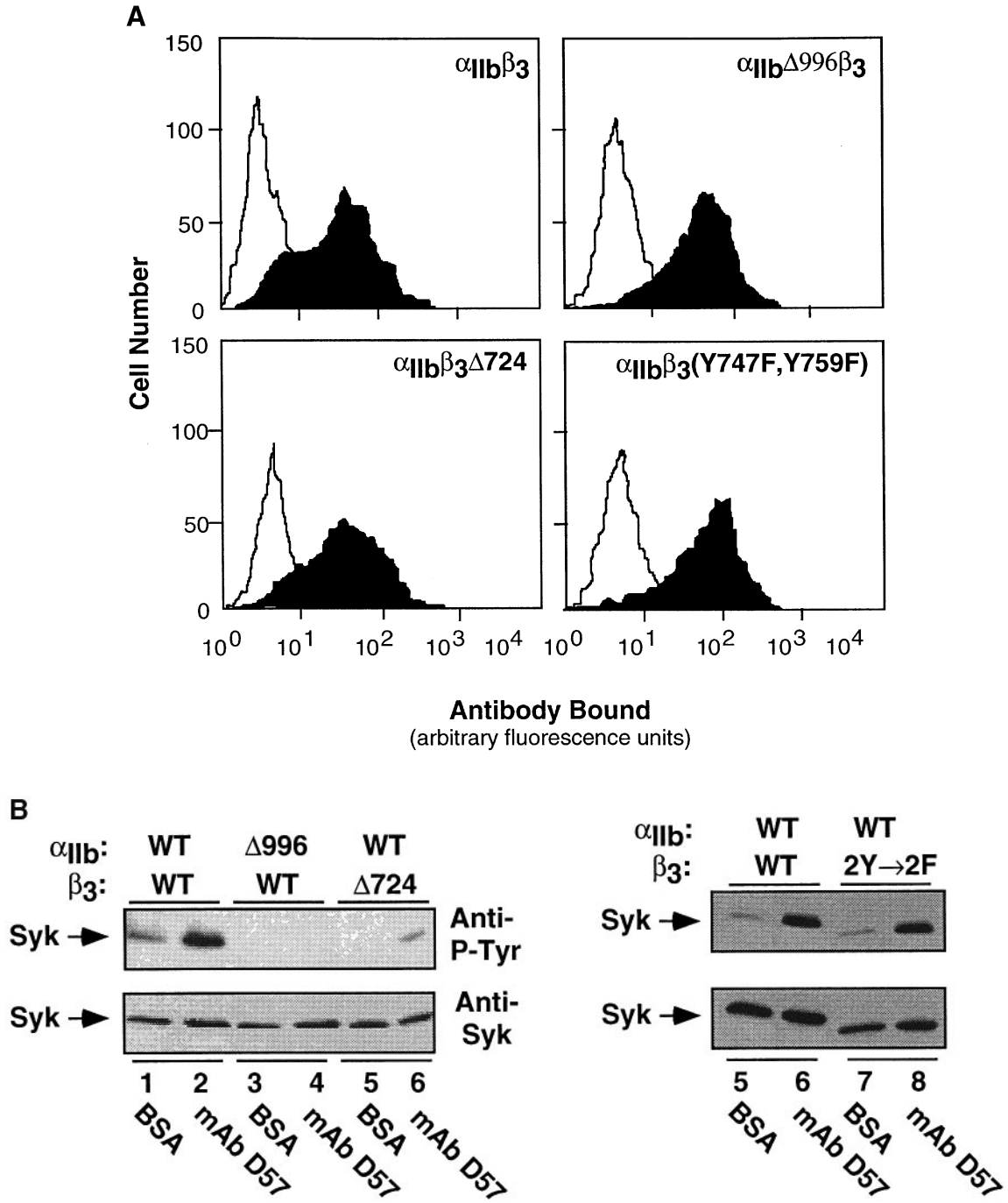

Fig. 6. Effect of deletions or mutations of the $\alpha_{IIb}\beta_3$ cytoplasmic tails on tyrosine phosphorylation of Syk. Syk was transfected into the indicated cell lines, which were studied 48 h later. In (**A**) surface expression of $\alpha_{IIb}\beta_3$ on 10 000 live cells was quantitated by flow cytometry using biotinylated mAb D57 and FITC-streptavidin (closed histograms). Open histograms represent the binding of FITC-streptavidin alone. In (**B**) each cell line was maintained in suspension or allowed to adhere to mAb D57 for 60 min. Then tyrosine phosphorylation of Syk was examined. The experiments shown in the left- and right-hand panels were conducted on separate occasions.

blots of CHO cells and it inhibited CD8/γ-induced tyrosine phosphorylation of Syk in these cells (Figure 7A).

A5 cells were then transfected with a Syk SH2(C) mutant, Syk(R195A), which is incapable of interacting with ITAMs. Despite this, Syk(R195A) became readily phosphorylated on tyrosine residues in response to A5 cell adhesion to fibrinogen or mAb D57, but it did not become phosphorylated in response to overexpression of CD8/γ (Figure 7B). These experiments establish that Syk activation by $\alpha_{IIb}\beta_3$ does not involve a classical Syk SH2/ITAM interaction.

Role of Syk autophosphorylation in $\alpha_{IIb}\beta_3$-dependent tyrosine phosphorylation of Syk

Tyrosine phosphorylation of Syk *in vivo*, whether triggered through immune response receptors or integrins, correlates closely with Syk activity measured *in vitro* (Clark *et al.*, 1994; Bu *et al.*, 1995; Kurosaki *et al.*, 1995; Shiue *et al.*,

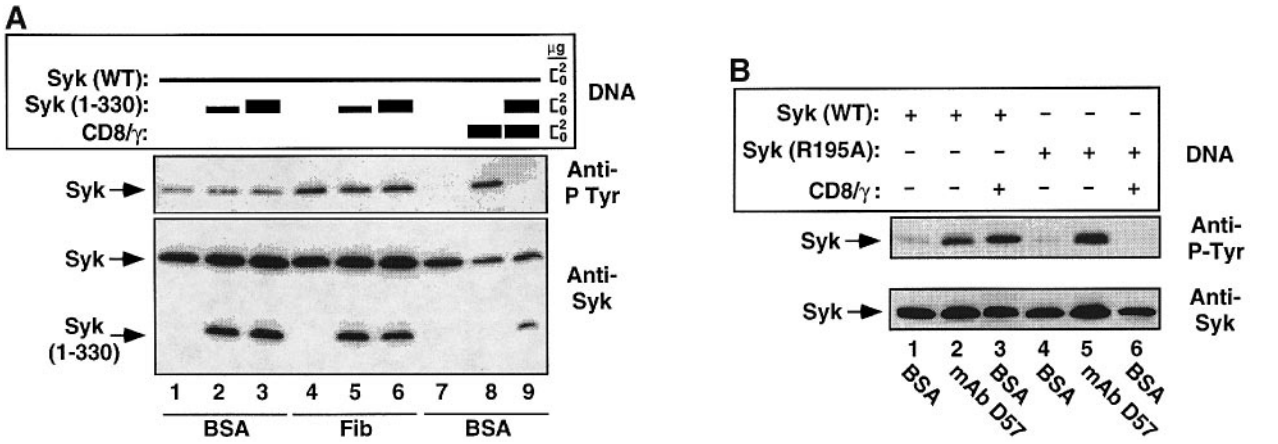

Fig. 7. Role of ITAMs in $\alpha_{IIb}\beta_3$-mediated tyrosine phosphorylation of Syk. In (**A**) A5 cells were transfected with the indicated amounts of wild-type Syk and truncated Syk(1-330) (lanes 1–6). Alternatively, native CHO cells were transfected with Syk, Syk(1-330) and a CD8/γ chimera (lanes 7–9). Forty-eight hours later, the cells were maintained in suspension (BSA) or allowed to attach to fibrinogen (Fib) for 60 min and tyrosine phosphorylation of Syk was determined. Also cell lysates (20 μg/lane) were probed on Western blots with anti-Syk antiserum to monitor expression of the relevant recombinant Syk proteins. Note that Syk(1-330) inhibited Syk tyrosine phosphorylation stimulated by CD8/γ (lane 8 versus lane 9) but not Syk phosphorylation stimulated by A5 cell adhesion to fibrinogen (lane 4 versus lanes 5 and 6) In (**B**) A5 cells were transfected as indicated with wild-type Syk (0.5 μg), kinase-inactive Syk(R195A) (0.5 μg) and CD8/γ (2 μg). Then Syk phosphorylation in response to cell adhesion to mAb D57 was assessed.

1995b). Similarly, increased tyrosine phosphorylation of Syk in adherent A5 cells was associated with increased Syk activity *in vitro* (not shown). In the case of immune response receptors, Syk activation appears to require the concerted action of a Src family kinase and autophosphorylation (Takata and Kurosaki, 1995; El-Hillal *et al.*, 1997). To determine whether Syk activation through $\alpha_{IIb}\beta_3$ involves autophosphorylation, A5 cells were transfected with wild-type or a kinase-inactive form of Syk(K402R) and assayed for Syk phosphorylation after adhesion to mAb D57. Unlike wild-type Syk, kinase-inactive Syk(K402R) failed to undergo tyrosine phosphorylation in adherent cells, indicating that activation of Syk through $\alpha_{IIb}\beta_3$ involves autophosphorylation (Figure 8A, compare lanes 2 and 9).

Role of Src in $\alpha_{IIb}\beta_3$-dependent tyrosine phosphorylation of Syk

Adhesion of fibroblasts, epithelial cells and NK cells to integrin substrates increases the activity of Src family kinases (Kaplan *et al.*, 1995; Rabinowich *et al.*, 1996; Schlaepfer and Hunter, 1997). Since CHO cells contain Src family members (Cary *et al.*, 1996), experiments were performed in A5 cells to examine their potential role in Syk activation through $\alpha_{IIb}\beta_3$. A5 cells were cotransfected with Syk and wild-type Src to determine whether overexpression of Src enhances $\alpha_{IIb}\beta_3$-induced Syk activation. These cells exhibited an ~15-fold increase in Src expression (assuming equivalent reactivity of the detecting antibody with the hamster and human proteins). The level of activated Src was also increased, as determined by Western blotting with an antibody specific for a phosphorylated tyrosine (Y416) in the Src activation loop (Figure 8A, lanes 3–6). Src overexpression and activation were associated with increased tyrosine phosphorylation of Syk, both in suspended and adherent cells. Nonetheless, Syk phosphorylation was still more pronounced in the adherent cells (Figure 8A, lane 3 versus lane 4). Src overexpression was also associated with adhesion-dependent tyrosine phosphorylation of kinase-inactive

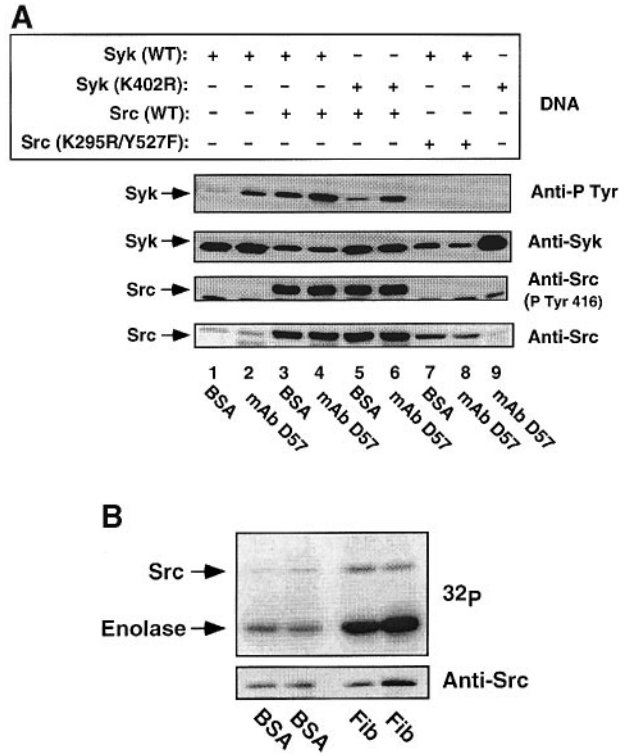

Fig. 8. Role of Src in $\alpha_{IIb}\beta_3$-dependent tyrosine phosphorylation of Syk. In (**A**) A5 CHO cells were cotransfected with the indicated plasmid DNAs and 48 h later Syk tyrosine phosphorylation in response to 60 min of cell adhesion to mAb D57 was studied. In addition, Src immunoprecipitates were analyzed on Western blots with an antibody specific for Src phosphotyrosine-416 (anti-Src P-Tyr 416) and 30 μg of protein from each cell lysate was analyzed for Src expression (anti-Src). There were some differences in the levels of Syk expression in this particular experiment, but this could not account for the differences observed in the anti-phosphotyrosine blots. In (**B**) A5 cells were transfected with Syk and 48 h later duplicate samples were incubated in suspension (BSA) or allowed to attach to fibrinogen for 15 min (Fib). The cells were then lysed and Src immunoprecipitates were subjected to *in vitro* kinase assay (^{32}P) and Src immunoblotting (anti-Src).

Syk(K402R), which is incapable of autophosphorylation (Figure 8A, lane 5 versus lane 6). Thus, Src can contribute to the process of integrin-mediated Syk activation, at least when it is overexpressed.

To evaluate the role of endogenous Src, A5 cells were cotransfected with Syk and an Src double mutant (K295R/Y527F), which is kinase-inactive and functions as a dominant-negative inhibitor of wild-type Src. Expression of Src(K295R/Y527F) abolished $\alpha_{IIb}\beta_3$-dependent tyrosine phosphorylation of Syk (Figure 8A, lanes 7 and 8). Under the same conditions, $\alpha_{IIb}\beta_3$-dependent tyrosine phosphorylation of epitope-tagged FAK was not affected (not shown). This suggests that Src activity is required for the induction of Syk phosphorylation by $\alpha_{IIb}\beta_3$. To determine whether Src is activated in A5 cells spread on fibrinogen, Src was immunoprecipitated from cell extracts and assayed for phosphorylation of the exogenous substrate, enolase (Figure 8B). The Src protein from cells attached to fibrinogen consistently displayed a 2- to 3-fold increase in autophosphorylation and phosphorylation of enolase. Taken together, the results in Figures 7 and 8 indicate that engagement of $\alpha_{IIb}\beta_3$ causes an increase in the catalytic activity of Src which is required for induction of tyrosine phosphorylation of Syk.

Discussion

The purpose of this study was to characterize the mechanism of Syk activation by the platelet integrin, $\alpha_{IIb}\beta_3$. Since Syk activation in platelets exhibits integrin-dependent and integrin-independent components, and platelets are not amenable to genetic manipulations *ex vivo*, we used a CHO cell model system. Several characteristic aspects of platelet integrin signaling could be reproduced in this system, and most relevant to this study was the $\alpha_{IIb}\beta_3$-dependent tyrosine phosphorylation of Syk and FAK. This permitted a detailed analysis of the functional relationships among $\alpha_{IIb}\beta_3$, Syk and FAK, and insight into how Syk is regulated by cell adhesion. Several major conclusions regarding the mechanism of Syk activation through $\alpha_{IIb}\beta_3$ can be drawn: (i) it depends on membrane-distal sequences in the cytoplasmic tails of both α_{IIb} and β_3; (ii) it does not depend on an SH2/ITAM interaction, unlike immune response receptor activation of Syk; (iii) it results from both Syk autophosphorylation and the action of a Src family kinase; (iv) it differs in several respects from FAK activation by $\alpha_{IIb}\beta_3$ such that a more proximal relationship between the integrin and Syk is suggested.

Integrin cytoplasmic tails have been shown to play important but poorly understood roles in many facets of integrin signaling (Sastry and Horwitz, 1993; Dedhar and Hannigan, 1996), and this study shows they are no less important for activation of Syk. Truncation of either α_{IIb} at residue 996 or β_3 at residue 724 eliminated the membrane-distal portions of the tails and abolished Syk phosphorylation by $\alpha_{IIb}\beta_3$ (Figure 6B). In contrast, truncation of the β_3 tail reduces adhesion-dependent phosphorylation of FAK, but truncation of the α_{IIb} tail does not (Leong *et al.*, 1995). Although one must be cautious in comparing results obtained with transfected human Syk and endogenous hamster FAK, these results indicate that integrins employ diverse mechanisms to regulate tyrosine kinases and the diversity begins at the level of the integrin cytoplasmic tails.

Why are the cytoplasmic tails of both α_{IIb} and β_3 needed for activation of Syk? One possibility is that both tails contribute residues that are directly involved in interaction with Syk or with a bridging molecule. Biophysical data indicate that α_{IIb} and β_3 cytoplasmic tail peptides interact with each other *in vitro* (Muir *et al.*, 1994; Haas and Plow, 1996), and similar interactions may take place in cells (Briesewitz *et al.*, 1996). Another possibility is that one cytoplasmic tail is needed to promote Syk tyrosine phosphorylation while the other prevents Syk dephosphorylation by a protein tyrosine phosphatase. Consistent with this, protein tyrosine phosphatases such as PTP-1B and SHP-1 become activated during platelet aggregation and redistribute to the $\alpha_{IIb}\beta_3$-rich core cytoskeleton (Frangione *et al.*, 1993; Ezumi *et al.*, 1995). A third possibility is that only the β_3 tail is directly involved in the mechanism of Syk activation but the α_{IIb} tail is needed to insure the correct subcellular juxtaposition of the β_3 tail with Syk. In fact, membrane-distal residues in α_{IIb} do regulate the recruitment of $\alpha_{IIb}\beta_3$ to focal adhesions (Ylanne *et al.*, 1993). It may be possible to resolve some of these possibilities by overexpressing isolated α_{IIb} or β_3 cytoplasmic tail chimeras, since these appear to be able to trigger some integrin signaling reactions and inhibit others (Chen *et al.*, 1994a; LaFlamme *et al.*, 1994; Lukashev *et al.*, 1994).

The modes of Syk activation through immune response receptors and integrins are distinctly different. In the former, ligand-induced receptor clustering stimulates tyrosine phosphorylation of receptor ITAMs by one or more Src family kinases, resulting in Syk engagement through the tandem SH2 domains (Daeron, 1997; Reth and Wienands, 1997). Platelets contain at least two ITAM-containing proteins, FcγRIIA and the FcRγ subunit, that in theory might be interposed between $\alpha_{IIb}\beta_3$ and Syk (Chacko *et al.*, 1996; Gibbins *et al.*, 1996). However, the present experiments rule out this possibility. First, a Syk fragment containing the tandem SH2 domains failed to inhibit $\alpha_{IIb}\beta_3$-mediated tyrosine phosphorylation of Syk, while the same fragment abolished ITAM-dependent Syk phosphorylation caused by overexpression of CD8/γ (Figure 7A). Second, an R195A mutation within the Syk SH2(C) domain had no effect on Syk phosphorylation through $\alpha_{IIb}\beta_3$, despite the fact that it abolished Syk phosphorylation through CD8/γ (Figure 7B). Finally, integrin activation of Syk was unaffected by simultaneous substitution of β_3 tail tyrosine residues 747 and 759 (Figure 6B), which have been shown *in vitro* to serve as docking sites for certain SH2 domains (Law *et al.*, 1996b).

Activation of Syk through immune response receptors involves Src family kinases (Kurosaki *et al.*, 1994; Scharenberg *et al.*, 1995); however Syk activation is not strictly dependent on Src kinases under all conditions (Kolanus *et al.*, 1993; Chu *et al.*, 1996; Latour *et al.*, 1997; Williams *et al.*, 1997; Zoller *et al.*, 1997). Src or a related kinase appears to be required for Syk activation by $\alpha_{IIb}\beta_3$ since a kinase-inactive variant of Src blocked $\alpha_{IIb}\beta_3$-induced Syk phosphorylation (Figure 8A). This inhibition does not appear to reflect a non-specific interference in $\alpha_{IIb}\beta_3$-mediated events since tyrosine phosphorylation of FAK was not inhibited by kinase-inactive

Src. The involvement of Src in Syk activation is further supported by the evidence presented here that Src catalytic activity was elevated following attachment of A5 CHO cells to fibrinogen and that overexpression of Src caused an increase in $\alpha_{IIb}\beta_3$-induced Syk phosphorylation. The finding that kinase-inactive Syk was not detectably phosphorylated in adherent A5 cells implies that the majority of Syk tyrosine phoshorylation is mediated by autophosphorylation.

We speculate that integrin ligation may activate Src and/or induce its redistribution within the cell so that it is in a position to initiate Syk activation. By analogy with T cell receptor activation of ZAP-70 (Qian and Weiss, 1997) and Fc receptor activation of Syk (El-Hillal *et al.*, 1997), integrins may trigger a Syk activation loop phosphorylation chain reaction that is initiated by Src and reinforced by Syk autophosphorylation. Previous data are consistent with the hypothesis that cell adhesion stimulates an interaction between Syk and Src or other Src family kinases. For example, they both redistribute to focal adhesions and to the Triton X-100-insoluble cytoskeletal fraction of adherent cells, including platelets (Grondin *et al.*, 1991; Clark and Brugge, 1993; Fox *et al.*, 1993; Kaplan *et al.*, 1995), and they co-precipitate from detergent lysates of activated leukocytes and platelets (Aoki *et al.*, 1995; Ozaki *et al.*, 1995; Couture *et al.*, 1996; Yan *et al.*, 1997). Platelets contain several Src family kinases (Huang *et al.*, 1991; Cary *et al.*, 1996), and further studies will be necessary to clarify the role of each in the Syk activation process.

This study illustrates that there are significant differences in the mechanisms by which $\alpha_{IIb}\beta_3$ regulates Syk and FAK. The binding of soluble fibrinogen to $\alpha_{IIb}\beta_3$ is sufficient to induce Syk phosphorylation in A5 cells and platelets. In contrast, additional post-ligand binding events during cell adhesion are needed for FAK phosphorylation in these cells (Figure 5) (Huang *et al.*, 1993; Clark *et al.*, 1994). Furthermore, Syk phosphorylation in adherent CHO cells and aggregated platelets is not abolished by cytochalasin D, but FAK phosphorylation is (Figure 4) (Clark *et al.*, 1994). Similarly, Syk phosphorylation triggered through β_2 integrins in monocytic cells is resistant to cytochalasins (Lin *et al.*, 1995). These findings are consistent with previous studies in many cell types showing that activation of FAK depends on signals from both integrins and agonist receptors that promote cytoskeletal assembly and reorganization (Shattil *et al.*, 1994; Guan and Chen, 1996; Rodríguez-Fernández and Rozengurt, 1996). This additional level of cytoskeletal organization may provide a scaffold for interactions between FAK and Src that are necessary for the optimal function of both proteins (Guan and Chen, 1996; Miyamoto *et al.*, 1996; Schlaepfer and Hunter, 1997).

The studies in both platelets and CHO cells point to a more proximal functional relationship between $\alpha_{IIb}\beta_3$ and Syk than between $\alpha_{IIb}\beta_3$ and FAK (Clark *et al.*, 1994). However, this does not necessarily mean there is a more proximal physical relationship. Indeed, FAK can bind directly to synthetic peptides mimicking β integrin cytoplasmic tails (Schaller *et al.*, 1995), and it becomes associated with clustered β_1 integrins in cells (Miyamoto *et al.*, 1995). However, it is not yet known if FAK and integrins interact directly *in vivo*. Syk can be co-precipitated with β_2 integrins from neutrophil lysates (Yan *et al.*, 1997), but it does not bind directly to a tyrosine-phosphorylated synthetic peptide mimicking the β_3 tail (Law *et al.*, 1996b) and it does not co-precipitate with $\alpha_{IIb}\beta_3$ from platelets or CHO cells (Law *et al.*, 1996b; J.Gao and S.J.Shattil, unpublished observations). Thus, the precise physical interactions that mediate early events in outside–in signaling remain to be determined. Nonetheless, since Syk apparently is not needed for FAK activation and vice versa, these two integrin-dependent protein tyrosine kinases may lie in parallel pathways downstream of $\alpha_{IIb}\beta_3$ rather than in a common pathway.

A major unresolved issue concerns the identity of substrates and effectors of Syk in an integrin signaling pathway. In the case of immune response receptor signaling, Syk is reported to interact with and phosphorylate or activate several proteins, including phospholipase Cγ (Law *et al.*, 1996a), c-Cbl (Ota *et al.*, 1996; Panchamoorthy *et al.*, 1996), Shc (Jabril-Cuenod *et al.*, 1996), Vav (Teramoto *et al.*, 1997), PI 3-kinase (Yanagi *et al.*, 1994) and SHIP (Crowley *et al.*, 1996). Experiments conducted in Syk knockout mice and Syk null cells indicate that this protein is necessary for normal survival, development and/or signaling of B lymphocytes, certain T lymphocytes and mast cells (Cheng *et al.*, 1995; Turner *et al.*, 1995; Costello *et al.*, 1996; Qin *et al.*, 1997). Syk activation through integrins may modulate some of these responses. For example, tyrosine phosphorylation in RBL-2H3 cells in response to occupancy of FcεRI is enhanced by adhesion of the cells to fibronectin (Hamawy *et al.*, 1993). Conversely, Syk activation through immune response receptors may modulate integrin-triggered cytoskeletal responses during adhesion of platelets and other hematopoietic cells. Of note in this regard, one downstream effector of Syk, Vav, possesses guanine nucleotide exchange activity for Rac (Teramoto *et al.*, 1997). In addition, Syk interacts with the actin-binding protein, cortactin (Maruyama *et al.*, 1997), and is required for ITAM-dependent F-actin assembly (Cox *et al.*, 1996). Further studies of recombinant Syk in the CHO cell model system and in Syk null hematopoietic cells should further our understanding of the function of this protein in integrin signaling.

Materials and methods

cDNAs, cell lines, antibodies and other reagents

The construction of expression vectors for human Syk and kinase-inactive Syk has been described (Zoller *et al.*, 1997). EMCV/Syk(1-330) is a variant of EMCV/Syk in which a stop codon was inserted after amino acid 330. The EMCV/Syk(R195A) mutant encodes an arginine-to-alanine mutation in the C-terminal SH2 domain of human Syk. Both mutants were made by site-directed mutagenesis (Kunkel, 1985). pRC-CMV/Src encodes wild-type murine c-Src. In pRC-CMV/Src(K295R/Y527F), the K295R mutation in the ATP-binding site renders the kinase inactive, and the Y527F mutation abolishes intramolecular interactions between the C-terminal tail and the SH2 domain. pSAP/CD8-γ encodes a chimera containing the extracellular and transmembrane domains of CD8 fused to the cytoplasmic domain of the γ subunit of FcεRI. EMCV/ZAP encodes wild-type human ZAP-70. pcDNA3 was from Invitrogen, Carlsbad, CA.

CHO cell lines that stably-express human wild-type or mutant $\alpha_{IIb}\beta_3$ have been described (Leong *et al.*, 1995; Hughes *et al.*, 1996). These include the A5 cell line (wild-type $\alpha_{IIb}\beta_3$) and three mutant cell lines [$\alpha_{IIb}\Delta996\beta_3$, $\alpha_{IIb}\beta_3\Delta724$ and $\alpha_{IIb}\beta_3$(Y747F,Y759F)]. Rabbit anti-Syk antiserum #0134 was raised against a synthetic peptide corresponding to a linear sequence in the interdomain B region of human Syk (residues

324–339; EPELAPWAADKGPQRE). Antiserum BC3, specific for $pp125^{FAK}$, was a gift from J.Thomas Parsons, University of Virginia (Schaller *et al.*, 1992). Antiserum specific for Src phosphotyrosine-416 was a gift from Andrew Laudano, University of New Hampshire. Antiserum specific for ZAP-70 was from Upstate Biotechnology, Lake Placid, NY. The murine monoclonal antibodies (mAb), mAb D57 (specific for $\alpha_{IIb}\beta_3$), anti-LIBS6 Fab (β_3 integrin subunit) and mAb 327 (Src) were characterized previously (Frelinger *et al.*, 1991; Clark and Brugge, 1993; Leong *et al.*, 1995). Monoclonal antibody 7E2 (β_1 integrin subunit) was a gift from Rudolph Juliano, University of North Carolina (Brown and Juliano, 1988). Monoclonal anti-phosphotyrosine antibodies 4G10 and PY20 were from Upstate Biotechnology and Transduction Laboratories (Lexington, KY), respectively. Monoclonal anti-Syk antibody 4D10 was from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Horse radish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin reagents were from Biosource International (Camarillo, CA) and Bio-Rad Laboratories (Hercules, CA), respectively.

Human fibrinogen was from Enzyme Research Laboratories, Inc., South Bend, IN. Human fibronectin was purified as described by Engvall (Engvall and Ruoslahti, 1977). Bovine serum albumin (BSA, fraction V) and sodium orthovanadate were from Fisher, Inc., Pittsburgh, PA. Protein A-Sepharose CL 4B and GammaBind Plus Sepharose were from Pharmacia Biotech, Piscataway, NJ. Pefabloc and aprotinin were from Boehringer Mannheim, Indianapolis, IN. Lipofectamine was from Gibco/BRL, Gaithersburg, MD. A bicinchoninic acid reagent for protein assay and SuperSignal reagent for Western blotting were from Pierce Chemical Co., Rockford, IL. Integrilin, a function-blocking cyclic peptide specific for $\alpha_{IIb}\beta_3$, was a gift from David Phillips, Cor Therapeutics, Inc. (Scarborough *et al.*, 1993). Ro 43–5054, a function-blocking peptidomimetic specific for $\alpha_{IIb}\beta_3$, was a gift from Beat Steiner, Basle, Switzerland (Alig *et al.*, 1992). All other reagents were from Sigma Inc., St. Louis, MO.

Transient expression of Syk and other proteins in CHO cells

CHO cells were grown to a 50–80% cell density in 100 mm culture dishes and transfected with the indicated amounts of plasmid DNA using Lipofectamine according to the manufacturer's protocol. When necessary, pcDNA3 was added to maintain total plasmid DNA at 4 μg. Six hours after transfection, the cells were washed and the medium replaced with 10 ml complete DME and 10% fetal calf serum. Thirty hours after transfection, the cells were washed again and the concentration of fetal calf serum was lowered to 0.5%.

Interaction of CHO cells with soluble and immobilized $\alpha_{IIb}\beta_3$ ligands

Forty-eight hours after transfection, CHO cells were trypsinized, washed twice with DME, resuspended to 3×10^6 cells/ml in DME and incubated for 45 min at 37°C with 20 μM cycloheximide. To test the effects of binding of soluble fibrinogen to $\alpha_{IIb}\beta_3$, suspended cells were incubated at 37°C in the presence of 250 μg/ml fibrinogen and 150 μg/ml anti-LIBS6, which binds to the β_3 subunit and increases the affinity of $\alpha_{IIb}\beta_3$ for fibrinogen (Frelinger *et al.*, 1991). Ro 43–5054 (18 μM) was added as a control to some tubes to specifically block fibrinogen binding (Alig *et al.*, 1992). After 15 min, the cells were washed with phosphate-buffered saline (PBS) and lysed in complete RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 10 mM Tris, pH 7.4, 1 mM Na_2EGTA, 1 mM sodium vanadate, 0.5 mM leupeptin, 0.25 mg/ml Pefabloc, 5 μg/ml aprotinin).

For studies of adherent cells, bacterial tissue culture plates were precoated with one of the following: 5 mg/ml BSA, 100 μg/ml fibrinogen, 10 μg/ml fibronectin, 10 μg/ml mAb D57 or 10 μg/ml mAb 7E2 (Leong *et al.*, 1995). After blocking for 2 h at room temperature with heat-denatured BSA, 1 ml of transfected cells at 3×10^6/ml was added to each plate and incubations were carried out at 37°C in a CO_2 incubator. After 60 min, non-adherent cells from BSA-coated plates were diluted 1:1 with PBS, sedimented at 100 *g* for 5 min and washed once with PBS before lysis in complete RIPA buffer. The adherent cells from plates coated with integrin ligands were rinsed twice with PBS, lysed on the plates with ice-cold complete RIPA buffer and scraped into microcentrifuge tubes. Lysates were incubated for 30 min on ice and clarified supernatants were processed for immunoprecipitation, Western blotting and *in vitro* kinase assays.

Immunoprecipitation, Western blotting and in vitro kinase assays

Equal amounts of protein from each lysate (typically 150–350 μg of protein, depending on the experiment) were immunoprecipitated with either 5 μl anti-Syk, anti-FAK or anti-ZAP-70 antiserum or 0.4 μg of the anti-Src mAb 327 as described in more detail previously (Huang *et al.*, 1993; Leong *et al.*, 1995). Precipitations were carried out in the presence of 15 μl BSA-blocked Protein A-Sepharose CL 4B beads for 60 min at 4°C. After washing the beads three times in ice-cold complete RIPA buffer, proteins were eluted in boiling Laemmli sample buffer containing 1 mM vanadate and 1% 2-mercaptoethanol. Proteins were separated on a 7.5% SDS–polyacrylamide gel and transferred to nitrocellulose (PROTRAN, Schleicher and Schuell, Keene, NH). Membranes were blocked with 6% non-fat dry milk, probed with the indicated primary and HRP-conjugated secondary antibodies, and immunoreactive bands were detected by enhanced chemiluminescence with development times of 0.1–1 min. To monitor loading of gel lanes, blots were stripped (2% SDS, 62.5 mM Tris, pH 6.7, 100 mM 2-mercaptoethanol for 30 min at 70°C) and reprobed with the appropriate antibodies. In some experiments, luminograms were scanned and labeled bands were quantitated by calibrated densitometry using a flatbed scanner, Macintosh 7300 computer and NIH Image software.

In vitro kinase activity in Syk immunoprecipitates was determined as described (Clark *et al.*, 1994). To measure kinase activity in Src immunoprecipitates, Syk-transfected A5 cells were incubated in BSA- or fibrinogen-coated plates for 15 min. Suspended and adherent cells were then lysed in buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl, pH 7.5, 2.5 mM sodium vanadate, 1 mM phenymethylsulfonyl fluoride, 0.5 mM leupeptin and 10 μg/ml aprotinin. Equal amounts of protein from each sample were then immunoprecipitated with anti-Src monoclonal antibody 327 and subjected to *in vitro* kinase assay and anti-Src immunoblotting as described (Clark and Brugge, 1996).

Flow cytometry

Cell surface expression of $\alpha_{IIb}\beta_3$ was quantitated by flow cytometry using biotinylated mAb D57 and FITC-streptavidin (Leong *et al.*, 1995).

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References

Alig,L., Edenhofer,A., Hadváry,P., Hürzeler,M., Knopp,D., Müller,M., Steiner,B., Trzeciak,A. and Weller,T. (1992) Low molecular weight, non-peptide fibrinogen receptor antagonists. *J. Med. Chem.*, **35**, 4393–4407.

Aoki,Y., Kim,Y.-T., Stillwell,R., Kim,T.J. and Pillai,S. (1995) The SH2 domains of Src family kinases associate with Syk. *J. Biol. Chem.*, **270**, 15658–15663.

Asselin,J., Gibbins,J.M., Achison,M., Lee,Y.H., Morton,L.F., Farndale, R.W., Barnes,M.J. and Watson,S.P. (1997) Collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase Cgamma2 in platelets independent of the integrin $\alpha_2\beta_1$. *Blood*, **89**, 1235–1242.

Bauer,J.S., Schreiner,C.L., Giancotti,F.G., Ruoslahti,E. and Juliano,R.L. (1992) Motility of fibronectin receptor-deficient cells on fibronectin and vitronectin: Collaborative interactions among integrins. *J. Cell. Biol.*, **116**, 447–487.

Briesewitz,R., Kern,A., Smilenov,L.B., David,F.S. and Marcantonio,E.E. (1996) The membrane-cytoplasm interface of integrin α subunits is critical for receptor latency. *Mol. Biol. Cell.*, **7**, 1499–1509.

Brown,P.J. and Juliano,R.L. (1988) Monoclonal antibodies to distinctive epitopes on the alpha and beta subunits of the fibronectin receptor. *Exp. Cell Res.*, **177**, 303–318.

Bu,J., Shaw,A.S. and Chan,A.C. (1995) Analysis of the interaction of ZAP-70 and syk protein-tyrosine kinases with the T-cell antigen receptor by plasmon resonance. *Proc. Natl Acad. Sci. USA*, **92**, 5106–5110.

Cambier,J.C. (1995) Antigen and Fc receptor signaling: The awesome power of the immunoreceptor tyrosine-based activation motif (ITAM) *J. Immunol.*, **155**, 3281–3285.

Cary,L.A., Chang,J.F. and Guan,J.L. (1996) Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *J. Cell Sci.*, **109**, 1787–1794.

Chacko,G.W., Duchemin,A.-M., Coggeshall,K.M., Osborne,J.M., Brandt,J.T. and Anderson,C.L. (1994) Clustering of the platelet Fcgamma receptor induces noncovalent association with the tyrosine kinase $p72^{syk}$. *J. Biol. Chem.*, **269**, 32435–32440.

Chacko,G.W., Brandt,J.T., Coggeshall,K.M. and Anderson,C.L. (1996) Phosphoinositide 3-kinase and $p72^{syk}$ noncovalently associate with the low affinity Fcgamma receptor on human platelets through an immunoreceptor tyrosine-based activation motif—Reconstitution with synthetic phosphopeptides. *J. Biol. Chem.*, **271**, 10775–10781.

Chan,A.C., Iwashima,M., Turck,C.W. and Weiss,A. (1992) ZAP-70: A 70 kDa protein-tyrosine kinase that associates with the TCR zeta chain. *Cell*, **71**, 649–662.

Chen,T. *et al.* (1996) The interaction of phosphorylated FcepsilonRIgamma immunoglobulin receptor tyrosine activation-based peptides with dual and single SH2 domains of $p72^{Syk}$. Assessment of binding parameters and real time binding kinetics. *J. Biol. Chem.*, **271**, 25308–25315.

Chen,Y.-P., O'Toole,T.E., Shipley,T., Forsyth,J., LaFlamme,S.E., Yamada,K.M., Shattil,S.J. and Ginsberg,M.H (1994a) 'Inside-out' signal transduction inhibited by isolated integrin cytoplasmic domains. *J. Biol. Chem.*, **269**, 18307–18310.

Chen,Y.-P., O'Toole,T.E., Ylänne,J., Rosa,J.-P. and Ginsberg,M.H. (1994b) A point mutation in the integrin β_3 cytoplasmic domain ($S^{752}\rightarrow P$) impairs bidirectional signaling through $a_{IIb}\beta_3$ (platelet glycoprotein IIb-IIIa). *Blood*, **84**, 1857–1865.

Cheng,A.M., Rowley,B., Pao,W., Hayday,A., Bolen,J.B. and Pawson,T. (1995) Syk tyrosine kinase required for mouse viability and B-cell development. *Nature*, **378**, 303–306.

Chu,D.H., Spits,H., Peyron,J.F., Rowley,R.B., Bolen,J.B. and Weiss,A. (1996) The Syk protein tyrosine kinase can function independently of CD45 or Lck in T cell antigen receptor signaling. *EMBO J.*, **15**, 6251–6261.

Clark,E.A. and Brugge,J.S. (1993) Redistribution of activated $pp60^{c-src}$ to integrin-dependent cytoskeletal complexes in thrombin-stimulated platelets. *Mol. Cell. Biol.*, **13**, 1863–1871.

Clark,E.A., Shattil,S.J., Ginsberg,M.H., Bolen,J. and Brugge,J.S. (1994) Regulation of the protein tyrosine kinase, $pp72^{syk}$, by platelet agonists and the integrin, $\alpha_{IIb}\beta_3$. *J. Biol. Chem.*, **46**, 28859–28864.

Clark,E.A. and Brugge,J.S. (1996) Tyrosine phosphorylation. In Watson,S. and Authi,K. (eds), *Platelet Function and Signal Transduction: A Practical Approach*. Oxford University Press, Oxford, UK.

Clark,E.A. and Brugge,J.S. (1995) Integrins and signal transduction pathways. The road taken. *Science*, **268**, 233–239.

Costello,P.S., Turner,M., Walters,A.E., Cunningham,C.N., Bauer,P.H., Downward,J. and Tybulewicz,V.L.J. (1996) Critical role for the tyrosine kinase Syk in signaling through the high affinity IgE receptor of mast cells. *Oncogene*, **13**, 2595–2605.

Couture,C., Deckert,M., Williams,S., Russo,F.O., Altman,A. and Mustelin,T. (1996) Identification of the site in the Syk protein tyrosine kinase that binds the SH2 domain of Lck. *J. Biol. Chem.*, **271**, 24294–24299.

Cox,D., Chang,P., Kurosaki,T. and Greenberg,S. (1996) Syk tyrosine kinase is required for immunoreceptor tyrosine activation motif-dependent actin assembly. *J. Biol. Chem.*, **271**, 16597–16602.

Crowley,M.T., Harmer,S.L. and DeFranco,A.L. (1996) Activation-induced association of a 145-kDa tyrosine-phosphorylated protein with Shc and Syk in B lymphocytes and macrophages. *J. Biol. Chem.*, **271**, 1145–1152.

Daeron,M. (1997) Fc receptor biology. *Annu. Rev. Immunol.*, **15**, 203–234.

Dedhar,S. and Hannigan,G.E. (1996) Integrin cytoplasmic interactions and bidirectional transmembrane signalling. *Curr. Opin. Cell. Biol.*, **8**, 657–669.

El-Hillal,O., Kurosaki,T., Yamamura,H., Kinet,J.P. and Scharenberg,A.M. (1997) Syk kinase activation by a src kinase-initiated activation loop phosphorylation chain reaction. *Proc. Natl Acad. Sci. USA*, **94**, 1919–1924.

Engvall,E. and Ruoslahti,E. (1977) Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer*, **20**, 1–5.

Ezumi,Y., Takayama,H. and Okuma,M. (1995) Differential regulation of protein-tyrosine phosphatases by integrin $\alpha_{IIb}\beta_3$ through cytoskeletal reorganization and tyrosine phosphorylation in human platelets. *J. Biol. Chem.*, **270**, 11927–11934.

Fox,J.E.B., Lipfert,L., Clark,E.A., Reynolds,C.C., Austin,C.D. and Brugge,J.S. (1993) On the role of the platelet membrane skeleton in mediating signal transduction. Association of GP IIb-IIIa, $pp60^{c-src}$, $pp62^{c-yes}$ and the $p21^{ras}$ GTPase-activating protein with the membrane skeleton. *J. Biol. Chem.*, **268**, 25973–25984.

Frangione,J.V., Oda,A., Smith,M., Salzman,E.W. and Neel,B.G. (1993) Calpain-catalyzed cleavage and subcellular relocation of protein phosphotyrosine phosphatase 1B (PTP-1B) in human platelets. *EMBO J.*, **12**, 4843–4856.

Frelinger,A.L.,III, Du,X., Plow,E.F. and Ginsberg,M.H. (1991) Monoclonal antibodies to ligand-occupied conformers of integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) alter receptor affinity, specificity and function. *J. Biol. Chem.*, **266**, 17106–17111.

Gibbins,J., Asselin,J., Farndale,R., Barnes,M., Law,C.L. and Watson,S.P. (1996) Tyrosine phosphorylation of the Fc receptor gamma-chain in collagen-stimulated platelets. *J. Biol. Chem.*, **271**, 18095–18099.

Grondin,P., Plantavid,M., Sultan,C., Breton,M., Mauco,G. and Chap,H. (1991) Interaction of $pp60^{c-src}$, phospholipase C, inositol-lipid and diacylglycerol kinases with the cytoskeletons of thrombin-stimulated platelets. *J. Biol. Chem.*, **266**, 15705–15709.

Guan,J.L. and Chen,H.C. (1996) Signal transduction in cell-matrix interactions. *Int. Rev. Cytol.*, **168**, 81–121.

Haas,T.A. and Plow,E.F. (1996) The cytoplasmic domain of $\alpha_{IIb}\beta_3$. A ternary complex of the integrin alpha and beta subunits and a divalent cation. *J. Biol. Chem.*, **271**, 6017–6026.

Haimovich,B., Lipfert,L., Brugge,J.S. and Shattil,S.J. (1993) Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands. *J. Biol. Chem.*, **268**, 15868–15877.

Hamawy,M.M., Mergenhagen,S.E. and Siraganian,R.P. (1993) Cell adherence to fibronectin and the aggregation of the high affinity immunoglobulin E receptor synergistically regulate tyrosine phosphorylation of 105–115-kDa proteins. *J. Biol. Chem.*, **268**, 5227–5233.

Huang,M.-M., Bolen,J.B., Barnwell,J.W., Shattil,S.J. and Brugge,J.S. (1991) Membrane glycoprotein IV (CD36) is physically associated with Fyn, Lyn and Yes protein tyrosine kinases in human platelets. *Proc. Natl Acad. Sci. USA*, **88**, 7844–7848.

Huang,M.-M., Lipfert,L., Cunningham,M., Brugge,J.S., Ginsberg,M.H. and Shattil,S.J. (1993) Adhesive ligand binding to integrin $\alpha_{IIb}\beta_3$ stimulates tyrosine phosphorylation of novel protein substrates before phosphorylation of $pp125^{FAK}$. *J. Cell. Biol.*, **122**, 473–483.

Hughes,P.E., Renshaw,M.W., Pfaff,M., Forsyth,J., Keivens,V.M., Schwartz,M.A. and Ginsberg,M.H. (1996) Suppression of integrin activation: A novel function of a Ras/Raf-initiated MAP-kinase pathway. *Cell*, **88**, 521–530.

Hynes,R.O. (1992) Integrins: versatility, modulation and signaling in cell adhesion. *Cell*, **69**, 11–25.

Ichinohe,T., Takayama,H., Ezumi,Y., Arai,M., Yamamoto,N., Takahashi,H. and Okuma,M. (1997) Collagen-stimulated activation of Syk but not c-Src is severely compromised in human platelets lacking membrane glycoprotein VI. *J. Biol. Chem.*, **272**, 63–68.

Jabril-Cuenod,B., Zhang,C., Scharenberg,A.M., Paolini,R., Numerof,R., Beaven,M.A. and Kinet,J.P. (1996) Syk-dependent phosphorylation of Shc—A potential link between Fc×RI and the Ras/mitogen-activated protein kinase signaling pathway through Sos and Grb2. *J. Biol. Chem.*, **271**, 16268–16272.

Juliano,R. (1996) Cooperation between soluble factors and integrin-mediated cell anchorage in the control of cell growth and differentiation. *BioEssays*, **18**, 911–917.

Kaplan,K.B., Swedlow,J.R., Morgan,D.O. and Varmus,H.E. (1995) c-Src enhances the spreading of *src–/–* fibroblasts on fibronectin by a kinase-independent mechanism. *Genes Dev.*, **9**, 1505–1517.

Keely,P.J. and Parise,L.V. (1996) The $\alpha_2\beta_1$ integrin is a necessary co-receptor for collagen-induced activation of Syk and the subsequent phosphorylation of phospholipase Cgamma2 in platelets. *J. Biol. Chem.*, **271**, 26668–26676.

Kimura,T., Sakamoto,H., Appella,E. and Siraganian,R.P. (1996) Conformational changes induced in the protein tyrosine kinase $p72^{syk}$ by tyrosine phosphorylation or by binding of phosphorylated immunoreceptor tyrosine-based activation motif peptides. *Mol. Cell. Biol.*, **16**, 1471–1478.

Kolanus,W., Romeo,C. and Seed,B. (1993) T cell activation by clustered tyrosine kinases. *Cell*, **74**, 171–183.

Kunkel,T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. USA*, **82**, 488–492.

Kurosaki,T., Takata,M., Yamanashi,Y., Inazu,T., Taniguchi,T., Yamamoto,T. and Yamamura,H. (1994) Syk activation by the Src-family tyrosine kinase in the B cell receptor signaling. *J. Exp. Med.*, **179**, 1725–1729.

Kurosaki,T., Johnson,S.A., Pao,L., Sada,K., Yamamura,H. and Cambier,J.C. (1995) Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling. *J. Exp. Med.*, **182**, 1815–1823.

LaFlamme,S.E., Thomas,L.A., Yamada,S.S. and Yamada,K.M. (1994) Single subunit chimeric integrins as mimics and inhibitors of endogenous integrin functions in receptor localization, cell spreading and migration and matrix assembly. *J. Cell Biol.*, **126**, 1287–1298.

Latour,S., Fournel,M. and Veillette,A. (1997) Regulation of T-cell antigen receptor signalling by Syk tyrosine protein kinase. *Mol. Cell. Biol.*, **17**, 4434–4441.

Law,C.-L., Chandran,K.A., Sidorenko,S.P. and Clark,E.A. (1996a) Phospholipase C-γ1 interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk. *Mol. Cell. Biol.*, **16**, 1305–1315.

Law,D.A., Nannizzi-Alaimo,L. and Phillips,D.R. (1996b) Outside–in signal transduction: $\alpha_{IIb}\beta_3$ (GP IIb-IIIa) tyrosine phosphorylation induced by platelet aggregation. *J. Biol. Chem.*, **271**, 10811–10815.

Leong,L., Hughes,P.E., Schwartz,M.A., Ginsberg,M.H. and Shattil,S.J. (1995) Integrin signaling: Roles for the cytoplasmic tails of $\alpha_{IIb}\beta_3$ in the tyrosine phosphorylation of $pp125^{FAK}$. *J. Cell Sci.*, **108**, 3817–3825.

Lin,T.H., Rosales,C., Mondal,K., Bolen,J.B., Haskill,S. and Juliano,R.L. (1995) Integrin-mediated tyrosine phosphorylation and cytokine message induction in monocytic cells. A possible signaling role for the Syk tyrosine kinase. *J. Biol. Chem.*, **270**, 16189–16197.

Lukashev,M.E., Sheppard,D. and Pytela,R. (1994) Disruption of integrin function and induction of tyrosine phosphorylation by the autonomously expressed β_1 integrin cytoplasmic domain. *J. Biol. Chem.*, **269**, 18311–18314.

Maruyama,S., Kurosaki,T., Sada,K., Yamanashi,Y., Yamamoto,T. and Yamamura,H. (1997) Physical and functional association of cortactin with Syk in human leukemic cell line K562. *J. Biol. Chem.*, **271**, 6631–6635.

Minoguchi,K., Benhamou,M., Swaim,W.D., Kawakami,Y., Kawakami,T. and Siraganian,R.P. (1994) Activation of protein tyrosine kinase $p72^{syk}$ by Fc×RI aggregation in rat basophilic leukemia cells. $p72^{syk}$ is a minor component but the major protein tyrosine kinase of pp72. *J. Biol. Chem.*, **269**, 16902–16908.

Miyamoto,S., Akiyama,S.K. and Yamada,K.M. (1995) Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science*, **267**, 883–885.

Miyamoto,S., Teramoto,H., Gutkind,J.S. and Yamada,K.M. (1996) Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: Roles of integrin aggregation and occupancy of receptors. *J. Cell Biol.*, **135**, 1633–1642.

Muir,T.W., Williams,M.J., Ginsberg,M.H. and Kent,S.B.H. (1994) Design and chemical synthesis of a neoprotein structural model for the cytoplasmic domain of a multisubunit cell-surface receptor: Integrin $\alpha_{IIb}\beta_3$ (platelet GPIIb-IIIa). *Biochemistry*, **33**, 7701–1108.

Müller,B., Cooper,L. and Terhorst,C. (1994) Molecular cloning of the human homologue to the pig protein-tyrosine kinase *syk*. *Immunogenetics*, **39**, 359–362.

Ota,Y., Beitz,L.O., Scharenberg,A.M., Donovan,J.A., Kinet,J.P. and Samelson,L.E. (1996) Characterization of Cbl tyrosine phosphorylation and a Cbl–Syk complex in RBL-2H3 cells. *J. Exp. Med.*, **184**, 1713–1723.

Ozaki,Y., Satoh,K., Kuroda,K., Qi,R., Yatomi,Y., Yanagi,S., Sada,K., Yamamura,H., Yanabu,M., Nomura,S. and Kume,S. (1995) Anti-CD9 monoclonal antibody activates $p72^{syk}$ in human platelets. *J. Biol. Chem.*, **270**, 15119–15124.

Panchamoorthy,G. *et al*. (1996) $p120^{cbl}$ is a major substrate of tyrosine phosphorylation upon B cell antigen receptor stimulation and interacts *in vivo* with Fyn and Syk tyrosine kinases, Grb2 and Shc adaptors and the p85 subunit of phosphatidylinositol 3-kinase. *J. Biol. Chem.*, **271**, 3187–3194.

Poole,A., Gibbins,J.M., Turner,M., van Vugt,M.J., van den Winkel,J.G.J., Saito,T., Tybulewicz,V.L.J. and Watson,S.P. (1997) The Fc receptor γ-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J.*, **16**, 2333–2341.

Qian,D.P. and Weiss,A. (1997) T cell antigen receptor signal transduction. *Curr. Opin. Cell Biol.*, **9**, 205–212.

Qin,S.F., Minami,Y., Hibi,M., Kurosaki,T. and Yamamura,H. (1997) Syk-dependent and -independent signaling cascades in B cells elicited by osmotic and oxidative stress. *J. Biol. Chem.*, **272**, 2098–2103.

Rabinowich,H., Manciulea,M., Herberman,R.B. and Whiteside,T.L. (1996) β_1 Integrin-mediated activation of focal adhesion kinase and its association with Fyn and Zap-70 in human NK cells. *J. Immunol.*, **157**, 3860–3868.

Reth,M. and Wienands,J. (1997) Initiation and processing of signals from the B cell antigen receptor. *Annu. Rev. Immunol.*, **15**, 453–479.

Rodríguez-Fernández,J.L. and Rozengurt,E. (1996) Bombesin, bradykinin, vasopressin and phorbol esters rapidly and transiently activate Src family tyrosine kinases in Swiss 3T3 cells—Dissociation from tyrosine phosphorylation of p125 focal adhesion kinase. *J. Biol. Chem.*, **271**, 27895–27901.

Rowley,R.B., Burkhardt,A.L., Chao,H.-G., Matsueda,G.R. and Bolen,J.B. (1995) Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Igα/Igβ immunoreceptor tyrosine activation motif binding and autophosphorylation. *J. Biol. Chem.*, **270**, 11590–11594.

Sastry,S.K. and Horwitz,A.F. (1993) Integrin cytoplasmic domains: Mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. *Curr. Opin. Cell Biol.*, **5**, 819–831.

Sastry,S.K. and Horwitz,A.F. (1996) Adhesion-growth factor interactions during differentiation: An integrated biological response. *Dev. Biol.*, **180**, 455–467.

Scarborough,R.M. *et al*. (1993) Design of potent and specific integrin antagonists. Peptide antagonists with high specificity for glycoprotein IIb-IIIa. *J. Biol. Chem.*, **268**, 1066–1073.

Schaller,M.D., Borgman,C.A., Cobb,B.S., Vines,R.R., Reynolds,A.B. and Parsons,J.T. (1992) $PP125^{FAK}$, a structurally unique protein tyrosine kinase associated with focal adhesions. *Proc. Natl Acad. Sci. USA*, **89**, 5192–5196.

Schaller,M.D., Otey,C.A., Hildebrand,J.D. and Parsons,J.T. (1995) Focal adhesion kinase and paxillin bind to peptides mimicking β integrin cytoplasmic domains. *J. Cell. Biol.*, **130**, 1181–1187.

Scharenberg,A.M., Lin,S., Cuenod,B., Yamamura,H. and Kinet,J.-P. (1995) Reconstitution of interactions between tyrosine kinases and the high affinity IgE receptor which are controlled by receptor clustering. *EMBO J.*, **14**, 3385–3394.

Schlaepfer,D.D. and Hunter,T. (1997) Focal adhesion kinase overexpression enhances Ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. *J. Biol. Chem.*, **272**, 13189–13195.

Schwartz,M.A., Schaller,M.D. and Ginsberg,M.H. (1995) Integrins: Emerging paradigms of signal transduction. *Annu. Rev. Cell Biol.*, **11**, 549–599.

Shattil,S.J., Haimovich,B., Cunningham,M., Lipfert,L., Parsons,J.T., Ginsberg,M.H. and Brugge,J.S. (1994) Tyrosine phosphorylation of $pp125^{FAK}$ in platelets requires coordinated signaling through integrin and agonist receptors. *J. Biol. Chem.*, **269**, 14738–14745.

Shiue,L. *et al*. (1995a) Interaction of $p72^{syk}$ with the gamma and β subunits of the high-affinity receptor for immunoglobulin E, FcεRI. *Mol. Cell. Biol.*, **15**, 272–281.

Shiue,L., Zoller,M.J. and Brugge,J.S. (1995b) Syk is activated by phosphotyrosine-containing peptides representing the tyrosine-based activation motifs of the high affinity receptor for IgE. *J. Biol. Chem.*, **270**, 10498–10502.

Takata,M. and Kurosaki,T. (1995) The catalytic activity of Src-family tyrosine kinase is required for B cell antigen receptor signaling. *FEBS Lett.*, **374**, 407–411.

Taylor,J.A., Karas,J.L., Ram,M.K., Green,O.M. and Seidel-Dugan,C. (1995) Activation of the high-affinity immunoglobulin E receptor Fc epsilon RI in RBL-2H3 cells is inhibited by Syk SH2 domains. *Mol. Cell. Biol.*, **15**, 4149–4157.

Teramoto,H., Salem,P., Robbins,K.C., Bustelo,X.R. and Gutkind,J.S. (1997) Tyrosine phosphorylation of the vav proto-oncogene product links FcεRI to the Rac-JNK pathway. *J. Biol. Chem.*, **272**, 10751–10755.

Turner,M. *et al*. (1995) Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature*, **378**, 298–302.

Wang,R., Shattil,S.J., Ambruso,D.R. and Newman,P.J. (1997) Truncation of the cytoplasmic domain of β_3 in a variant form of Glanzmann thrombasthenia abrogates signaling through the integrin $\alpha_{IIb}\beta_3$ complex. *J. Clin. Invest*., in press.

Williams,S., Couture,C., Gilman,J., Jascur,T., Deckert,M., Altman,A. and Mustelin,T. (1997) Reconstitution of T cell antigen receptor-induced Erk2 kinase activation in Lck-negative JCaM1 cells by Syk. *Eur. J. Biochem.*, **245**, 84–90.

Yamada,K.M. and Geiger,B. (1997) Molecular interactions in cell adhesion complexes. *Curr. Opin. Cell Biol.*, **9**, 76–85.

Yan,S.R., Huang,M. and Berton,G. (1997) Signaling by adhesion in human neutrophils—Activation of the p72^{syk} tyrosine kinase and formation of protein complexes containing p72^{syk} and Src family kinases in neutrophils spreading over fibrinogen. *J. Immunol.*, **158**, 1902–1910.

Yanabu,M. *et al*. (1997) Tyrosine phosphorylation and p72^{syk} activation by an anti-glycoprotein Ib monoclonal antibody. *Blood*, **89**, 1590–1598.

Yanagi,S., Sada,K., Tohyama,Y., Tsubokawa,M., Nagai,K., Yonezawa,K. and Yamamura,H. (1994) Translocation, activation and association of protein-tyrosine kinase (p72^{syk}) with phosphatidylinositol 3-kinase are early events during platelet activation. *Eur. J. Biochem.*, **224**, 329–333.

Ylanne,J., Chen,Y., O'Toole,T.E., Loftus,J.C., Takada,Y. and Ginsberg, M.H. (1993) Distinct functions of integrin-alpha and integrin-beta subunit cytoplasmic domains in cell spreading and formation of focal adhesions. *J. Cell Biol.*, **122**, 223–233.

Zoller,K.E., MacNeil,I.A. and Brugge,J.S. (1997) Protein tyrosine kinases Syk and ZAP-70 display distinct requirements for Src family kinases in immune response receptor signal transduction. *J. Immunol.*, **158**, 1650–1659.

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